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Abstract

In this study, we investigated the changes of 13 trace metal and metalloid concentrations (i.e. Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, V, Zn) and their subcellular fractionation in juvenile cuttlefish *Sepia officinalis* reared in controlled conditions between hatching and 2-months post-hatching. In parallel, metallothionein concentrations were determined. Our results highlighted contrasting changes of studied metals. Indeed, As and Fe concentrations measured in hatchlings suggested a maternal transfer of these elements in cuttlefish. The non-essential elements Ag and Cd presented the highest accumulation during our study, correlated with the digestive gland maturation. During the 6 first weeks of study, soluble fractions of most of essential trace metals (i.e. Co, Cr, Cu, Fe, Se, Zn) slowly increased consistently with the progressive needs of cuttlefish metabolism during this period.
In order to determine for the first time in a cephalopod how metal concentrations and their subcellular distributions are impacted when trace metal exposed, we studied previously described parameters in juveniles exposed to dissolved Zn at environmental (i.e. 50 µg l-1) and sublethal (i.e. 200 µg l-1) levels. Moreover, oxidative stress (i.e. glutathione S-transferases (GST), superoxide dismutase (SOD), catalase, lipid peroxidation (LPO)) was assessed in digestive gland and gills after 1 and 2 months exposures. Our results highlighted no or low ability of this stage of life to regulate dissolved Zn accumulation during all studied period, consistently with high sensitivity of this organism. Notably, Zn exposures caused a dose-dependent Mn depletion in juvenile cuttlefish, and an increase of soluble fraction of Ag, Cd, Cu without accumulation modifications, suggesting substitution of these elements (i.e. Mn, Ag, Cd, Cu) by Zn. In parallel, metallothionein concentrations decreased in individuals the most exposed to Zn. Finally, no perturbations in oxidative stress management were detected in gills, whereas level modifications of GST, SOD and catalase activities were recorded in digestive gland, resulting in LPO content increase after 6-week exposure at low

Zn concentration. Altogether, these perturbations are consistent with previously described high sensitivity of juvenile cuttlefish towards Zn. Our results underlined the need to study deeply contamination impact on this animal at this stage of life.

Keywords: mollusk cephalopod; juvenile stage; zinc; metal subcellular distribution; oxidative stress; *Sepia officinalis*

Highlights

- Important changes of metal concentrations occurred in cuttlefish after hatching.
- During the 6-weeks post-hatching, metallothionein synthesis was important.
- Juvenile cuttlefish showed low ability to regulate dissolved zinc accumulation.
- Perturbations of Ag, Cd, Cu and Mn regulation were induced by zinc exposure.
- 52 µg l^{-1} zinc exposure caused oxidative stress in the cuttlefish digestive gland.

Introduction

Mollusk cephalopods are known for their importance in trophic marine ecosystems both as predator and prey (Chouvelon et al., 2011; Clarke, 1996). The economic importance of their fisheries has recently grown worldwide to compensate for finfish stock depletion (Jereb et al., 2005), whereas abundant literature underlines their sensitivity to a wide range of environmental parameters (reviewed in Pierce et al., 2008). Despite growing anthropogenic pressures on marine ecosystems, few studies investigated the potential physiological impacts of contaminants on these organisms (Di Poi et al., 2014, 2013; Lacoue-Labarthe et al., 2010a, 2009c; Sen and Sunlu, 2007). Yet, early life stages (i.e. eggs and juveniles) of economically important cephalopods are likely to be impacted by anthropogenic contaminants because they develop in coastal areas where these compounds are found at relatively high concentrations (Colas, 2011; Pierce et al., 2010). This is the case of the common cuttlefish *Sepia officinalis*, which is characterized by inshore migration during spring and summer months to reproduce and spawn, and juvenile residence in coastal areas from 1 to 2 months post-hatching to take advantage of prey abundance (Bloor et al., 2013; Boucaud-Camou and Boismery, 1991; Le Goff and Daguzan, 1991). In spite of the shielding properties of the eggshell against metals (Bustamante et al., 2006c, 2004, 2002b; Lacoue-Labarthe et al., 2011, 2010b, 2009b, 2008b), cuttlefish embryos are impacted by low metal concentration exposure (Lacoue-Labarthe et al., 2010a, 2009a). In addition, accumulation in soft tissues of waterborne Ag, Cd, and Zn followed by relatively long-term retention was reported in juveniles (Bustamante et al., 2004, 2002b). Surprisingly little is known about the effects of such trace metal exposure on juvenile cuttlefish physiology (Lacoue-Labarthe et al., 2010a, 2009a). [1]

[1]*Abbreviations:* CDNB: 1-chloro-2,4-dinitrobenzene; DPP: differential pulse polarographic analysis; GSH: reduced glutathione; GST: glutathione S-transferase; HMWP: high molecular weight proteins; LPO: lipid peroxidation; MDA: malondialdehyde; MT: metallothionein; PBS: phosphate buffered saline; ROS: reactive oxygen species; SOD: superoxide dismutase.

Despite a short life cycle of 1 to 2 years, *Sepia officinalis* (as other cephalopods) is known to accumulate various contaminants (e.g. Ansari et al., 2012; Bustamante et al., 2006a, 2006b, 1998; Danis et al., 2005; Miramand and Bentley, 1992). Bioaccumulation of trace metals such as Ag, Cd, Cu, Fe and Zn has been reported in the digestive gland, sometimes at very high concentrations (e.g. Bustamante et al., 2006a; Decleir et al., 1978; Martin and Flegal, 1975; Miramand and Bentley, 1992; Schipp and Hevert, 1978). Notably, trace metal accumulation starts after hatching and takes place through the entire life cycle, yet tissue-specific accumulation depends on digestive gland maturation (Miramand et al., 2006). While several studies highlighted the essential role of the digestive gland in the detoxification of trace metal elements in *Sepia officinalis* and other cephalopods (Bustamante et al., 2008, 2004, 2002b; Lacoue-Labarthe et al., 2009c; Martin and Flegal, 1975; Miramand and Bentley, 1992; Raimundo et al., 2014, 2010), no study investigated metal subcellular fractionation at juvenile stages during maturation of this organ (i.e. the first month post-hatching).

Detoxification mechanisms of marine invertebrates mainly involve the precipitation of metals into insoluble concretions and the binding to cytosolic proteins, both being interconnected (Wallace et al., 2003; Wang and Rainbow, 2010). Metallothioneins (MTs) are one class of these metal-binding proteins, characterized by low molecular weight, high cystein content and heat stability. They play a role in the homeostasis of the essential metals, Cu and Zn, but can be induced by various other metals (e.g. Ag, Cd, Hg) that they bind (Amiard et al., 2006; Cosson, 1991; Wang and Rainbow, 2010). In cephalopods, subcellular distribution of metals in the digestive gland appears to be species- and element-specific, but most studies reported mainly association of Ag, Fe, Mn, Pb and Zn with the insoluble fraction, while Cd, Co and Cu are mainly associated with the cytosolic fraction (Bustamante et al., 2006a, 2002a; Finger and Smith, 1987; Tanaka et al., 1983). Cu is also the main element found associated with MTs in the digestive gland of several cephalopod species (Bustamante et al., 2006a;

Finger and Smith, 1987; Tanaka et al., 1983), whereas results about Ag- and Cd-MT association remain controversial (Bustamante et al., 2006a, 2002a; Finger and Smith, 1987; Tanaka et al., 1983). To date, the MT involvement in the homeostasis of the cephalopod juvenile stage has been investigated once in the squid *Loligo forbesii* without highlighting its significant role in Cu and Zn management (Craig and Overnell, 2003).

Although Zn is an essential nutrient of living organisms – it is a component of more than 300 enzymes and other proteins (McCall et al., 2000), some studies highlighted its toxicity in marine organisms at concentrations of 100 µg l^{-1} and above (e.g. Amado Filho et al., 1997; Brereton et al., 1973; Nadella et al., 2013; Tellis et al., 2014; Watling, 1982). In European marine waters, dissolved Zn is relatively concentrated, regularly measured near 10 µg l^{-1} (Lachambre and Fisson, 2007a; Sheahan et al., 2007), and worldwide, some studies reported concentrations reaching several hundred µg Zn l^{-1} in anthropically impacted bays, gulfs and estuaries (Amado Filho et al., 1997; Liu and Wang, 2013; Srinivasa Reddy et al., 2005). Zn is also one of the most concentrated metals in the digestive gland of cuttlefish (Bustamante et al., 2006a; Miramand and Bentley, 1992; Miramand et al., 2006; Rjeibi et al., 2014). Its effects on the uptake of other metals, their subcellular distribution, and MT concentrations have been highligthed in bivalve mollusks (Blackmore and Wang, 2002; Hennig, 1986; Lemoine et al., 2000; Liu and Wang, 2013; Shi and Wang, 2004). For instance, Zn exposure induced an increase in Zn uptake as well as that of Cd and Cu in the bivalve *Crassostrea hongkongensis* (Liu and Wang, 2013). To the best of our knowledge, such responses have not been assayed in cephalopods. Finally, trace metals are known for their ability to induce reactive oxygen species (ROS) production, causing oxidative stress as previously described in bivalves (e.g. Company et al., 2004; Funes et al., 2006; Geret and Cosson, 2002; Geret et al., 2002), gastropods (Chandran et al., 2005; Malanga et al., 2004; Radwan et al., 2010) and the cephalopod *Octopus vulgaris* (Semedo et al., 2012). To avoid or

counteract ROS effects, organisms use a set of enzymes including glutathione S-transferases (EC 2.5.1.18, GST), superoxide dismutase (EC 1.15.1.1, SOD) and catalase (EC 1.11.1.6). Their activities have been extensively used as biomarkers of oxidative stress, and correlated to the presence of metals in invertebrates (e.g. Regoli and Principato, 1995; Semedo et al., 2012; Vlahogianni et al., 2007). The imbalance between production and removal of ROS may result in lipid peroxidation (LPO), a biomarker often used to evaluate the degree of oxidative stress (e.g. Di Salvatore et al., 2013; Radwan et al., 2010; Vlahogianni et al., 2007; Zielinski and Pörtner, 2000).

In order to better describe and understand metal regulation in juvenile cuttlefish and point out the effect of physiological modifications on the bioaccumulation and detoxification processes associated with digestive gland maturation, we quantified the presence, changes of the concentrations and the subcellular distributions of 13 elements (Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, V, and Zn) in juvenile cuttlefish during the period corresponding to their coastal life stage (i.e. first 2 months post-hatching). The concentration of MTs was also assayed and correlated with metal concentration modifications. Concurrently, these parameters were measured under Zn-exposure, in addition to oxidative stress parameters (GST, SOD, catalase, and LPO) in the digestive gland and in the gills which are the tissue directly in contact with food and waterborne trace elements, respectively.

1. Materials and methods

2.1. Animals

Cuttlefish eggs were collected from traps set along the Calvados coast (Basse-Normandie, France) during summer 2012. All rearing procedures were held at the Centre de Recherches en Environnement Côtier (Luc-sur-Mer, France). At the laboratory, the eggs were separated for optimal oxygenation, acclimatized at 17 ± 1°C, and maintain in open-circuit

tanks until hatching. Between 1 and 2 day post-hatching, juveniles were randomly acclimatized in experimental structures and fed 24 h later according to hatchling vitellus consumption period (Yim and Boucaud-Camou, 1980). During all experiment, cuttlefish were fed once a day with alive brown shrimp *Crangon crangon* fished every 2 days in front of the marine station. In order to obtain homogeneous intra- and inter-batch growth, the number of prey was adapted to the cuttlefish size and special attention was payed daily to feed all animals (in control as well as Zn-exposed conditions) with shrimps of the same size range.

2.2. Experimental design

2.2.1. Experimental system and seawater quality follow-up

To allow individual follow-up, constant exposure, as well as long-term water quality (particularly considering nitrogen compounds), we set up experimental units described below. In a 40-l tank (corresponding to one experimental unit), seawater mixing and oxygenation were provided by 2 water pumps (NJ400, Aquarium systems, France) and 2 air stones. A constant water renewal was provided in each experimental unit by peristaltic pump (521F/D2, Watson-Marlow Pumps Group, France), supplied with frequency converter and pumpheads (312X2, Watson-Marlow Pumps Group, France), at a rate of 30 ml min^{-1} from 60-l reservoirs. Water used during all experiments was decanted charcoal-filtered natural seawater to minimize particles and other dissolved metals attendance. Depending on renewal rate, reservoirs were refilled each day with seawater (either Zn-contaminated or control). In order to avoid animal interactions and to allow individual follow-up, a compartmentalized sieve was used. Each compartment had an area of 104 cm^2, which is enough space for optimal development of single juvenile according to Forsythe et al. (1994). During all experiments, seawater parameter monitoring was checked daily. Parameter variations for all experimental structures were: temperature: 17.5 ± 0.5°C; salinity: 32.5 ± 1 psu; ammonia-, nitrite-, and

nitrate-nitrogen below 0.5 mg l^{-1}, 0.5 mg l^{-1}, and 80 mg l^{-1}, respectively (according to Oestmann et al. (1997)); pH: 8.0 ± 0.1; and dissolved oxygen: > 9 mg l^{-1}. Experiments were realized under natural photoperiod. To keep high water quality, feces were siphoned every 3 days in order to reduce juvenile disturbance.

2.2.2. Zn-exposures and measurements

During summer 2012 and according to previous studies on mortality induced by dissolved Zn on juvenile cuttlefish (Le Pabic et al., under review), juvenile cuttlefish were exposed to 50 and 200 µg Zn l^{-1} (respectively, Z1 and Z2) for 8- and 6-weeks, respectively, alongside a control. Both Zn concentrations were environmentally realistic, with Z2 corresponding to concentration close to the previously determined mortality threshold value for 2-week post-hatching juvenile cuttlefish (Le Pabic et al., under review). The exposure media were spiked daily just after reservoir water renewal, using 20.84 g l^{-1} $ZnCl_2$ (i.e. 10.0 g Zn l^{-1}) stock solution ($ZnCl_2$ reagent grade, ≥ 98%, Sigma-Aldrich, France), prepared in 0.22 µm-filtered artificial seawater (25.5 g NaCl, 6.4 g $MgSO_4$, 5.2 g Hepes, 1.5 g $CaCl_2 \cdot 2H_2O$, 0.75 g KCl in 1 liter Milli-Q water, pH 7.4). According to preliminary trials, each reservoir was spiked weekly with respective nominal concentration plus 10% in order to keep constant concentrations and compensate animal absorption and experimental unit adsorption.

For all 3 batches (control and the 2 exposure conditions), 20 ml of seawater was sampled daily, immediately acidifed with 1% nitric acid (HNO_3 69.5%, Normapur grade), and stored at 4°C until analyses. Zn concentrations in the seawater were determined as previously described in Devos et al. (2012). Triplicate analyses were done on each sample and the mean values were used for evaluation. The limit of detection, defined as the background plus three times the standard deviation of the blank (3σ), for the determination of the Zn concentration in seawater was 6.2 µg l^{-1}.

2.2.3. Sampling procedure

All samplings were performed on 24-h fasting animals. Considering the elemental concentration assays, 9 animals were sampled at the beginning of the experiment, thereafter 5 animals from each batches were randomly removed every 2-weeks exposure (i.e. after 2-, 4-, 6- and 8-weeks). According to organ size, samplings dedicated to enzymatic assays were realized with 5 animals per conditions after 4-week exposure in Z2, and 4-, 6- and 8-week exposure in control and Z1 conditions. All animals sampled were anesthetized in solution of 2% ethanol with 17.5 mg l^{-1} $MgCl_2$ in seawater for 5 min, afterwards they were measured (weight and mantle length). Individuals dedicated to elemental analysis were immediately frozen in liquid nitrogen, whereas the digestive glands and the gills were sampled on animals with cephalopodium (i.e. head, arms and funnel) preliminary removed (Di Poi et al., 2014). Tissues were then frozen in liquid nitrogen and stored at -80°C for enzymatic assay, whereas whole animals were freeze-dried and individually ground for MTs and trace element analysis.

2.3. Subcellular fractionation and MT determination

Whole cuttlefish were analysed for their MT levels. Between 30 and 100 mg of ground dry tissue were homogenized on ice in 4-6 ml (depending of aliquot size) 100 mM Tris buffer with β-mercaptoethanol at pH 8.1 and then centrifuged (30 000 *g*; 30 min; 4°C). Around 100 µl of supernatants were used for MT assay, while pellets (i.e. the insoluble fraction) and the remaining supernatant (i.e. the soluble fraction) were stored frozen for further trace element analysis. The supernatant aliquot was then submitted to protocol described in Lucia et al. (2012) in order to obtain heat-stable protein fraction. Finally, differential pulse polarographic analysis (DPP) was used to determine the amount of MTs in this fraction and set up as described in Lucia et al. (2012). Results are expressed in µg of MTs per g of dry homogenized

tissue (µg g^{-1} dw).

2.4. Trace element analysis in respective soluble and insoluble fractions

For the analysis of all elements (i.e. Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, V and Zn), from 50 to 300 mg of each subcellular fractions were digested following protocol described in Metian et al. (2008).

Trace elements were analysed by Inductively Coupled Plasma Atomic Emission Spectrometry (Varian Vista-Pro ICP-OES) and Mass Spectrometry (ICP-MS II Series Thermo Fisher Scientific). Reference tissues – dogfish liver DOLT-4 (NRCC), lobster hepatopancreas TORT-2 (NRCC) – were treated and analysed in the same way as the samples. Results were in line with the certified values, and the standard deviations were low, proving good repeatability of the method. The results for standard reference materials displayed recovery of the elements ranging from 73% to 114%. The detection limits (µg g^{-1} dw) were 0.015 (Cd), 0.017 (Ag), 0.02 (Co, Cr, Pb), 0.03 (Ni), 0.08 (Mn), 0.1 (Cu, Se), 0.2 (As), 0.3 (V) and 3.3 (Fe and Zn). Trace element concentrations are expressed in µg g^{-1} dw.

2.5. Biochemical analysis

Sampled digestive glands and gills of one animal were separately weighed before to be homogenized with a Potter-Elvehjem homogeniser in ice-cold phosphate buffered saline (500 mM NaCl, 10 mM Na_2HPO_4 and 3.1 mM KH_2PO_4, pH 7.4, PBS) containing 1% Halt Protease Inhibitor Cocktail, EDTA-Free (100X, Thermo Fisher Scientific, Waltham, USA). Weight/volume ratio of 1/2 and 1/1 was used for digestive gland and gills, respectively. Homogenates were then centrifuged at 12,500 *g* for 15 min at 4°C (S9 fraction), and supernatants used for determining protein content, LPO, as well as enzymatic activities (GST,

SOD, catalase). All these analyses were adapted for microplate and realized in triplicate for each individual.

2.5.1. Protein assay

Total protein concentration was determined according to the Bradford method (1976) using bovine serum albumin (Sigma-Aldrich Chemicals, France) as standard. Enzymatic activities and LPO were expressed in relation to protein concentration measured in S9 fraction.

2.5.2. GST assay

GST activity was measured according to the Habig et al. method (1974). Briefly, 50 µl of S9 fraction adjusted to 250 µg prot ml^{-1} in PBS were mixed with 200 µl of reagent solution (1 mM reduced glutathione (GSH), 10 mM Hepes, 125 mM NaCl, and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), pH 6.5). The enzymatic reaction kinetic was spectrophotometrically monitored at 340 nm for 20 min at 25°C. The results were expressed as µmol of glutathione-CDNB conjugates per min and mg of proteins (µmol min^{-1} mg^{-1} prot).

2.5.3. SOD assay

SOD activity was measured using the assay developed by Paoletti et al. (1986) and adapted for microplate. A SOD standard (Sigma-Aldrich Chemicals, France) range from 2.5 to 10 U ml^{-1} was made in PBS. The inhibition of ß-NADH (Sigma-Aldrich Chemicals, France) oxidation was then monitored at 340 nm for 90 min at 25°C using 20 µl of S9 fraction adjusted to 150 µg prot ml^{-1} in PBS, or SOD standard, mixed with 173 µl of 0.35 mM ß-NADH, 6 mM EDTA and 3 mM $MnCl_2$, and 20 µl of 10 mM β-mercaptoethanol (Sigma-Aldrich Chemicals, France). The results were expressed in U mg^{-1} of protein.

2.5.4. Catalase assay

Catalase activity was monitored using the method previously described by Babo and Vasseur (1992). A catalase standard (Sigma-Aldrich Chemicals, France) range from 1 to 6 U ml^{-1} was made in PBS. Then, 100 µl of S9 fraction (adjusted respectively to 1.5 and 1.0 mg prot ml^{-1} in PBS for digestive gland and gills) or catalase standard were mixed with 100 µl of hydrogen peroxyde 28 mM. The hydrogen peroxyde degradation kinetics were assessed at 240 nm for 15 min at 25°C in UV microplate (Greiner Bio-One, Germany), and the results were expressed in U mg^{-1} of protein.

2.5.5. LPO determination

Lipid peroxidation level was assessed via malondialdehyde (MDA) content determined using a commercially available MDA assay kit (Oxis International, USA). The method was based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA at 45°C. The blue product was quantified by measuring absorbance at 586 nm (Gérard-Monnier et al., 1998). The results were expressed in nmol MDA g^{-1} of protein.

2.6. Statistical analysis

To study the changes of each variables function of time, their homogeneity of variances (Levene test) and normal residual distributions (Shapiro test) were first tested. According to the results, ANOVA or permutational ANOVA (in case of non normal residuals) followed by non parametric pairwise permutational *t*-test ($N<30$) were realized. In case of variance non-homogeneity, Kruskal-Wallis test followed by the Wilcoxon signed-rank were used. In some cases, logarithmic (Log; As total concentration) or reciprocal (Fe total concentration) were used to meet the underlying assumptions of normality and homogeneity

of variances. Pearson and Spearman correlations, respectively between soluble and insoluble concentrations of trace metals, were analyzed according to data binormality assumption. Comparisons between variables from control and Zn exposed individuals were realized using ANCOVA when data profiles were linear (i.e. MTs, Mn total concentration, GST activity). In the case of MT comparison, a square transformation was used to realized this test. Otherwise, comparison with control was assessed at each time-point using Student's *t*-test. R software and packages were used for statistics and graphics (Fox and Weisberg, 2011; Hervé, 2012; R Core Team, 2012).

3. Results

No mortality occured during this experiment.

3.1. Trace metal in juvenile cuttlefish

In this study, the soluble and insoluble amounts of trace metals as well as the MT content were measured in each animal.

3.1.1. Variation of the trace metal concentration

In order to study metal regulation in healthy juveniles, the concentration of 13 trace elements was assayed in whole juvenile cuttlefish directly after hatching and at 2-, 4-, 6- and 8-week post-hatching (Table 1). Trace element concentrations were grouped together according to their changes in whole juvenile cuttlefish between hatching time and 8 weeks. During this period, the total concentration of 5 elements decreased (As, Cr, Fe, Mn and Ni), while it increased for 4 elements (Ag, Cd, Cu and Se), remained constant for 3 elements (Co, Pb and Zn) and stayed below the detection limit for V. Among decreasing elements, As and Fe displayed the highest concentrations found in the hatchlings (176 ± 76 and 103 ± 71 µg g^{-1} dw, respectively), whereas Cr, Mn and Ni displayed the lowest concentrations of 1.87 ± 0.36, 2.67 ± 0.36 and 1.71 ± 0.46 µg g^{-1} dw, respectively. This decreasing phase mainly occurred

between hatching time and the 6th week for all these elements, reaching 55.8 ± 9.4, less than 0.28, 25.3 ± 4.1, 1.95 ± 0.1 and 1.06 ± 0.08 µg g^{-1} dw, respectively for As, Cr, Fe, Mn and Ni. Among increasing elements, the Cu increase occurred during the 2 first weeks (from 47.2 ± 5.0 to 59.3 ± 3.1 µg g^{-1} dw) afterwards its concentration remained constant. Although increasing, Se concentration did not present clear pattern of change during the period (ranging between 1.81 ± 0.21 and 2.64 ± 0.05 µg g^{-1} dw). During the first month of the study, the non-essential elements Ag and Cd presented an important accumulation from values lower than 0.1 µg g^{-1} dw in hatchlings for both elements to 2.09 ± 0.22 and 0.39 ± 0.06 µg g^{-1} dw, respectively at 1 month post-hatching, afterwards their concentrations remained stable. During all the studied period, Co, Pb and Zn concentrations remained constant around 0.21 ± 0.04, 0.41 ± 0.12 and 88.3 ± 7.8 µg g^{-1} dw, respectively.

3.1.2. Variation of the subcellular distribution

To better understand the change of subcellular fractions of trace metals in juveniles during the first 2 months post-hatching, soluble trace elements were assayed as the proportion of the total content between hatching and 2 month post-hatching in whole animals (Table 2). During this period, the subcellular distributions of As, Cd, Mn, Ni and Pb remained constants at around 93.0 ± 1.5, 28.2 ± 4.2, 36.5 ± 7.8, 75.9 ± 6.0 and 77.9 ± 5.5 % in the soluble fraction, respectively. Soluble Ag distribution alone decreased significantly, from 10.4 ± 1.7 to 7.4 ± 0.6 % between 2 and 6 weeks. In contrast, the distribution of soluble Co, Cu, Fe and Zn increased mainly between hatching and 6 weeks from 49.2 ± 5.9 to 74.9 ± 2.0, 27.3 ± 11.8 to 55.1 ± 5.1, 14.0 ± 12.8 to 48.6 ± 7.7 and 14.8 ± 5.8 to 26.6 ± 4.0 %, respectively. Our study highlighted that Ag, Cd, Fe, Mn and Zn were mainly associated to the insoluble fraction while As, Co, Cr, Ni, Pb and Se were mainly found in the soluble fraction in the whole juvenile cuttlefish. Notably, Cu was the only element displaying a major proportion as insoluble form

in the hatchlings, subsequently switching to a majority as soluble form at the end of the study period. The positive correlations between the soluble fractions of all trace elements (Table 3) as well as the insoluble fractions (Table 4) allowed at distinguishing 2 groups of elements: Ag, Cd, Co, Cu, and Zn on one side and As, Cr, Fe, Mn, and Ni on the other side. Otherwise, soluble and insoluble concentrations of Pb as well as Se fell into different correlation groups.

3.1.3. Variation of MT concentration and correlation with soluble element concentrations

The MT concentrations were assayed in the heat-stable soluble fraction of whole juveniles during the first 2 months post-hatching (Fig. 1). Its concentrations increased dramatically along the experiment, highlighting an important MT synthesis occurred at this period, particularly during the 6 first weeks post-hatching, which increased from 686 ± 93 to 5551 ± 631 µg g^{-1} dw. During this period, MT concentrations and size (weight and mantle length) had a positive linear correlation (r=0.93 and 0.90, respectively, and *p*-values < 0.001). After the 6^{th} week, MT concentrations decreased back to 4-week levels (i.e. 3617 ± 417 µg g^{-1} dw).

Positive correlations were found between MT concentrations and soluble Cu and Zn concentrations (r=0.41 and ρ=0.47, respectively) (Table 3).

3.2. Zn exposures

Table 5 shows the mean Zn concentrations during studied period (i.e. 8 and 6 weeks, respectively for Z1 and Z2) expressed in µg l^{-1} and µM, measured in the seawater of control, Z1 and Z2 batches. Table 6 shows the mean concentrations of the studied trace elements in the brown shrimp used as food during the study.

3.2.1. Elemental concentration perturbations

The variation of Zn concentrations in juvenile cuttlefish following the exposure to the waterborne metal is presented in Figure 2. Whereas Zn concentrations remained constant in control (i.e. < 6.2 µg l^{-1}), Zn concentration increased in both conditions with stabilization occurring in Z1 (i.e. 52 µg l^{-1}) after 4-week exposure at 302 ± 27 µg g^{-1} dw, and a constant increase in Z2 (i.e. 184 µg l^{-1}) reaching 959 ± 132 µg g^{-1} dw after 6-week exposure (Fig. 2).

Apart from Zn, the only element showing a variation in total concentration was Mn (Fig. 3). Thus, a dose-dependent decrease of Mn concentration occurred after 2- and 6-week exposure in Z2 and Z1 conditions, respectively. ANCOVA analysis highlighted a similar profile (i.e. slope) in the 3 conditions studied but at different concentration levels (i.e. y-intercept) for both Zn-exposures (*p*-value < 0.01 and 0.001 for Z1 and Z2, respectively).

3.2.2. Variation of subcellular distribution

The exposure to waterborne Zn induced a general increase of the proportion of soluble Ag, Cd, Cu and Zn (Fig. 4). In Z2, all these elements presented a significant soluble percentage increase regardless of the exposure time of around 2.2-, 2.0-, 1.2- and 1.4-fold the control level for Ag, Cd, Cu and Zn, respectively. Notably, their profiles remained similar to those of the control, and only soluble proportions appeared modified. In Z1, similar increase (towards control condition) of the soluble proportions were measured after 2-week exposure for Ag, Cd and Zn, for all elements after 4-week exposure, whereas the soluble proportion of each of these elements was similar to those of the control after 6-week exposure. During the 2 last weeks exposure, whereas Cd, Cu and Zn soluble proportion increased again, Ag remained similar to the level in the control.

3.2.3. MT perturbations

According to the linear profile of MT concentration observed in the control and to the lack of differences between Z1 and the control conditions after 8-week exposure (data not shown), the general change of this parameter was assayed during the first 6-week period. ANCOVA analysis highlighted no significant difference on MT concentrations at Z1 (*p*-value = 0.17) compared to control, and significant negative effect at Z2 considering all studied period (i.e. from 0 to 6-week exposure; *p*-value = 0.009) (Fig. 5).

3.2.4. Oxidative stress in digestive gland and gills

Because of the small size of organs and the lack of digestive gland differentiation (in rudimentary form during the first month post-hatching), first samplings were realized after 1-month exposure in order to allow individual analysis.

In gills, no effect of Zn exposures on GST, SOD and catalase activities or LPO level were detected, regardless of the exposure time (data not shown). In contrast, some important modifications of these parameters were measured in the digestive gland. Indeed, a significant increase in GST activity was observed in this organ after 4-week exposure to Z2 (Fig. 6) and in Z1 (ANCOVA *p*-value = 0.026). Notably, GST activity also increased in the digestive glands of control individuals during the sampling period. SOD activities increased at each sampling date in Z1 alone (Fig. 6), whereas catalase activities significantly decreased after 4-week exposure in Z2, and 6- and 8-week exposure in Z1 (Fig. 6). Finally, LPO presented higher levels after 6- and 8-week exposure in Z1 (Fig. 7). Notably, LPO level in the digestive gland of the control decreased between 4- and 6-week post-hatching.

4. Discussion

According to French national monitoring of inshore seawater, the values of dissolved metal concentrations in natural seawater used during this experiment were similar or lower

than national median concentration except for Ag, with a concentration 4 times higher than national median (ranged 2-4 ng l^{-1}; Boust et al., 2004; Chiffoleau et al., 2001; Etourneau et al., 2013; Ifremer, 2007; Lachambre and Fisson, 2007b). In order to minimize contamination from both particle and dissolved trace elements, seawater was decanted before being charcoal-filtered.

4.1. Metal concentrations in juvenile cuttlefish

Hatchlings presented higher trace element concentrations higher than previously reported in cephalopods for As, Cr, Fe, Mn and Ni (Bustamante et al., 2008; Horowitz and Presley, 1977; Ichihashi et al., 2001a, 2001b; Kojadinovic et al., 2011; Miramand and Bentley, 1992; Napoleão et al., 2005; Raimundo et al., 2014, 2010). Among them, As, Fe and to a lesser extent, Cr, displayed the highest concentrations followed by an important decrease during the first 6-weeks post-hatching (Table 1). According to the very important mean content of As and Fe in hatchlings – 176 and 103 µg g^{-1} dw, respectively – and to their low dissolved concentrations in our rearing seawater – around 1 and 5 µg l^{-1}, respectively (Boust et al., 2004; Lachambre and Fisson, 2007c) –, it is very likely that maternal transfer is the main source for both elements. As and Fe accumulation was previously reported in cephalopod genital tissues: the oviduct gland of the giant squid *Architeuthis dux* is the tissue containing the highest As concentration (Bustamante et al., 2008), and high Fe concentrations were found in the genital tract of female cuttlefish from the English channel (173 ± 14 µg g^{-1} dw) as well as in eggshells from the same location (398 ± 64 µg g^{-1} dw; Miramand and Bentley, 1992; Miramand et al., 2006). In contrast, low Fe concentration was previously reported in *S. officinalis* hatchlings from the Mediterranean sea, suggesting an important environmental influence on this process (Villanueva and Bustamante, 2006). Maternel transfer is also likely to be the main source of Cr found in hatchlings. Indeed, higher Cr

concentrations were found in juveniles of the squid *Stenoteuthis oualaniensis* than in adults (Ichihashi et al., 2001a), and higher Cr concentrations were reported in ovary and genital tract of *A. dux* and *S. officinalis* than in other tissues (Bustamante et al., 2008; Miramand and Bentley, 1992). The important decrease in total concentration of As, Fe and Cr during the 6 first weeks post-hatching suggested excretion of these elements at this period (Table 1).

In spite of their low total concentrations in hatchlings, Mn and Ni values (2.67 and 1.71 µg g^{-1} dw, respectively) were higher than previously reported in whole adult *S. officinalis* (Miramand and Bentley, 1992). Because of these low numerical values and the previously documented ability of Mn to accumulate in cuttlefish embryos through the eggshell (Lacoue-Labarthe et al., 2010b), but not through maternal transfer (Lacoue-Labarthe et al., 2008a), high Mn and Ni likely bioaccumulated from seawater during the egg development. Consistently, the dissolved concentrations of Mn and Ni in our rearing seawater was in the upper range of national median range (i.e. around 7 and 2 µg l^{-1} of Mn and Ni, respectively; Boust et al., 2004; Chiffoleau et al., 2001). The slow and constant total concentration decreases observed during the first 6 weeks post-hatching was very likely the result of dilution by growth (Rainbow and White, 1989) (Fig. 1).

Elements that strongly accumulated in our study were the non-essential elements Ag and Cd, with around 10- and 4-fold increase, respectively, during the first month post-hatching. Interestingly, their concentrations stabilized in subsequent time-points (Table 1), correlating with the time of digestive gland maturation (Yim and Boucaud-Camou, 1980). Previous studies showed that the mature cuttlefish digestive gland contains more than 70 % of the total body content in both elements (Bustamante et al., 2002b; Miramand and Bentley, 1992; Miramand and Guary, 1980; Miramand et al., 2006). Once in this tissue, Ag is quickly excreted whereas Cd is stored in a less toxic form, mainly associated to cytosolic proteins, allowing to its long-term retention at very high concentrations in the digestive gland

(Bustamante et al., 2002a, 2002b). Interestingly, the major difference in accumulation processes of these elements between juvenile with non-mature digestive gland and adult is the higher assimilation efficiency by juveniles of contaminants contained in food, as juvenile digestion is mostly intracellular (Bustamante et al., 2004, 2002b; Yim and Boucaud-Camou, 1980).

The only element with a decrease in its soluble fraction during our study was the non-essential trace metal Ag. This decrease occured during the first 6 weeks post-hatching, underlying the set-up of an efficient detoxification pathway of Ag and correlating with the digestive gland maturation which is involved in Ag insolubilisation (Bustamante et al., 2006a; Martoja and Marcaillou, 1993) (Table 2).

Other elements with a modification of their subcellular fractionation were the essential trace metals Co, Cr, Cu, Fe, Se and Zn, with an increase in their soluble proportion (Table 2). All increases occurred during the first 4- or 6-weeks post-hatching and were consistent with important growth of cuttlefish and essential roles of these elements, such as hemocyanin synthesis for Cu, vitamin B_{12} production for Co and glutathion peroxidase activity for Se.

The determination of positive correlations between soluble and insoluble concentrations revealed two distinct groups. The first group comprises Ag, Cd, Co, Cu and Zn, all of which are known to highly accumulate in the cephalopod digestive gland and to have affinity for sulfur-containing proteins such as MTs (Amiard et al., 2006; Vašák, 1991). In contrast, the second group is composed of trace elements that do not accumulate in the digestive gland: As, Cr, Mn and Ni are homogeneously allocated in cephalopod tissues and Fe accumulates in other organs (Bustamante et al., 2008, 2000; Kojadinovic et al., 2011; Miramand and Bentley, 1992; Miramand and Guary, 1980; Napoleão et al., 2005; Raimundo et al., 2014, 2010; Schipp and Hevert, 1978; Ueda et al., 1979) (Tables 3 and 4).

The only elements with soluble fraction positively correlated with MT concentrations in our study were Cu and Zn, consistently with the major role of MTs in homeostasis of both elements (e.g. Amiard et al., 2006; Cosson, 1991; Roesijadi, 1992). These correlations are also consistent with the potential role of MTs in the detoxification process of non-essential metals such as Ag and Cd (Table 3).

4.2. Zn exposures

The Zn accumulation observed during our experiment highlighted the decreasing ability of juvenile cuttlefish to regulate the Zn-buildup when exposed to dissolved concentrations equal or above 52 ± 3 µg l^{-1} (Z1; Fig. 2). Notably, this treshold appeared low considering Zn accumulation in other marine organisms such as the crustaceans *Palaemon elegans* and *Carcinus maenas* (Rainbow, 1985; White and Rainbow, 1982) and the bivalve *Perna viridis* (Chan, 1988) which regulated Zn buildup at concentrations above 100 µg l^{-1}. Nevertheless, metal uptake appears strongly species specific (e.g. Rainbow, 2002; Wang and Rainbow, 2005). The stabilization of Zn concentration occurring in Z1 animals after 4-week exposure (around 302 ± 27 µg g^{-1} dw) likely resulted from an equilibrium state between water and concentrations reached in animals rather than from a regulatory process (Fig. 2). Indeed, in Z2 conditions, Zn accumulation was observed at all time-points, whereas regulatory processes in other aquatic organisms lead to maintenance of internal concentrations close to control levels (Chan, 1988; Rainbow, 1985; White and Rainbow, 1982).

Apart from this Zn accumulation, a Zn dose-dependent depletion of total Mn in animals was observed during Zn exposures without fractionation or profile concentration changes (Fig. 3). To the best of our knowledge, such depletion of Mn concentrations due to Zn exposure was not previously reported. According to the similar chemical properties of Mn^{2+} and Zn^{2+} ions, Mn substitution by Zn appears likely: these elements are both used in

enzymes as Lewis acids (Bock et al., 1999; McCall et al., 2000) and their divalent ions have almost the same radius (0.75 Å for Mn^{2+} and 0.74 Å for Zn^{2+}; Bock et al., 1999). In addition, Mn and Zn are borderline metals without binding preferences for oxygen, nitrogen and sulfure ligands, and as such are found in the two main types of metal rich granules in mollusks: those rich in phosphate salts, and those rich in sulfure salts (Chvapil, 1973; Marigómez et al., 2002; Niebœr and Richardson, 1980; Pan and Wang, 2012). Our study suggests Zn binding to Mn ligands at high dissolved Zn concentrations (Z1 and above). Because such Mn^{2+} replacement is most often observed with Mg^{2+}, Mg homeostasis could be an important parameter to survey under Zn perturbation (Bock et al., 1999).

The increase in soluble Ag-, Cd- and Cu fractions observed in Zn-exposed juveniles (Fig. 4) differed from those found in similar studies on bivalves which reported some modifications in metal accumulation due to new ligand synthesis (e.g. MTs, GSH), thus allowing higher metal burden (e.g. Liu and Wang, 2014, 2013). In our study, no accumulation modifications were observed, except for Zn, in spite of modified subcellular distributions, thus suggesting the absence of Zn-induced synthesis of new ligand (Mouneyrac et al., 2002). We suggest that insoluble ligands of Ag, Cd, and Cu become occupied by significantly accumulated Zn, resulting in the observed increase of their respective soluble fractions. Such increases would have to be regulated by soluble ligands such as MTs, GSH and/or high molecular weight proteins (HMWP) to avoid the production of ROS and their associated damages. According to the positive correlation found between MTs and Zn in control juveniles, the different biological half-life of MT depending of their cation linkage and the previously reported shorter half-life of Zn-thioneins, we also suggest that the lack of MTs increase in Z1 and their decrease in Z2 (Fig. 5) may result from their intensive use by Zn resulting on progressive depletion of the MTs cuttlefish stock (Amiard et al., 2006; Cosson, 1991). Alternatively, this decrease may result from important MT degradation after metal

transport into lysosomes for metal rich granule formation (Liu and Wang, 2013; Mouneyrac et al., 2002). The management of Ag, Cd, Cu and Zn could also be assured by other cytosolic molecules such as the small tripeptide glutathion (GSH) which is one of the most abundant sulfure ligands found in most cells (Dickinson and Forman, 2002), and/or HMWP (Serafim and Bebianno, 2010). Consistently with this hypothesis, a study describing subcellular fractions of metals in the cuttlefish digestive gland highlighted that a part of cytosolic Cd, Cu and Zn were linked with compounds of size range similar to GSH and HMWP, whereas cytosolic Ag was detected only associated with compounds of HMWP size range (Bustamante et al., 2006a).

Oxidative stress bioassays indicated a lack of gill sensitivity to Zn exposure, suggesting the absence of Zn accumulation in cephalopod gills, unlike previously reported in bivalves (Company et al., 2010, 2008; Funes et al., 2006). In contrast, all biomarkers were affected by Zn exposure in the digestive gland, highlighting increased oxidative stress and reduced regulation of ROS resulting from Zn accumulation. First, the increase in GST activity after 1 and 2 month-exposure to Z2 and Z1 (Fig. 6), respectively, was consistent with an increase in free metal binding as the GST enzyme conjugates GSH with a great variety of electrophilic compounds such as metal ions to avoid ROS production (Dickinson and Forman, 2002). A possible consequence of this elevated GST activity is the depletion in free GSH, further increasing free metal concentrations (Dickinson and Forman, 2002). Second, the SOD activity increase measured after 4-, 6- and 8-week exposure to Z1 highligthed an increased ROS production under the superoxide anion form ($O_2^{\cdot -}$), which is consistent with a saturation of the metal-ligation system. SOD activity reduces $O_2^{\cdot -}$ – the first synthetized oxygen free-radicals, into H_2O_2 (Halliwell and Gutteridget, 1984; Janknegt et al., 2007), which is then decomposed into H_2O and O_2 by catalase (Wang et al., 2013). The catalase activity decrease observed after 6- and 8-week exposure in Z1 contrasts with the increased SOD activity and

suggests an imbalance in ROS management. This decreased catalase activity may result from previously described inhibitory effects of Zn, Cd and/or Cu on this enzyme (Chandran et al., 2005; Company et al., 2004; Geret et al., 2002). The increase in LPO in Z1 after 6-week exposure confirmed such imbalance occuring simultaneously to catalase decrease (Fig. 7). Nevertheless, no effect was observed before this period, whereas increases in Ag-, Cd- and Zn cytosolic concentrations were detected starting at 2 weeks of Zn exposure, or 4 weeks in the case of Cu, suggesting a progressive decrease in the regulation of ROS production.

Our results highlighted the great sensitivity of juvenile cuttlefish to dissolved Zn exposure. For the first time in a cephalopod, modification of metal concentrations as well as subcellular distributions by a single metal exposure is highlighted. According to these perturbations, our results suggest a low plasticity of this stage of life to regulate the effects of medium dissolved Zn exposure such as oxidative stress. The synthesis of new ligands to control accumulation of this element appeared very limited. Moreover, we studied the ability to control the ROS production in several tissues in a juvenile cephalopod. Contrarily to those of bivalves, cuttlefish gills presented a lower Zn accumulation potential compare to digestive gland. In this tissue, damages were recorded after 6-week exposure in 52 µg l^{-1} – a concentration lower or of the same order than values registered in coastal environments (Lachambre and Fisson, 2007a; Sheahan et al., 2007) and gulfs or estuaries (Amado Filho et al., 1997; Di Salvatore et al., 2013; Liu and Wang, 2013; Popham and D'Auria, 1982; Srinivasa Reddy et al., 2005), worldwide. Further studies on other pollutants will be necessary to characterize the breadth of cuttlefish sensitivity to anthropogenic contamination and confirm its impact on cuttlefish recruitment, as previously suggested by Gras (2013).

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Table 1: Trace element concentrations (mean ± SD; µg g^{-1} dw) in whole juvenile *Sepia officinalis* during its two first months of life.

Life stage	Mantle length (mm)	Age	*N*	Ag	As	Cd	Co	Cr
Hatchlings	9.2 ± 0.5	0 days	9	< 0.14 [c]	176 ± 76 [a]	< 0.10 [c]	0.20 ± 0.06	1.87 ± 0.36 [a]
	8.4–9.8			< 0.10–0.15	113–346	< 0.06–0.21	0.14–0.35	1.35–2.28
Juveniles	10.3 ± 0.1	14 days	5	1.57 ± 0.18 [b]	139 ± 33 [a]	0.25 ± 0.03 [b]	0.20 ± 0.02	1.58 ± 0.46 [a]
	10.1–10.5			1.37–1.86	112–192	0.22–0.30	0.18–0.24	0.93–2.11
	11.7 ± 0.7	1 month	5	2.09 ± 0.22 [a]	95.2 ± 17.1 [b]	0.39 ± 0.06 [a]	0.24 ± 0.03	0.45 ± 0.12 [b]
	10.9–12.7			1.79–2.34	73.2–113	0.33–0.46	0.20–0.27	0.34–0.63
	16.4 ± 0.9	1.5 months	5	1.92 ± 0.21 [a]	55.8 ± 9.4 [c]	0.28 ± 0.06 [ab]	0.21 ± 0.02	< 0.28 [c]
	15.0–17.4			1.74–2.27	42.2–68.1	0.23–0.37	0.19–0.24	< 0.20–0.28
	22.3 ± 1.3	2 months	5	1.97 ± 0.09 [a]	50.4 ± 4.0 [c]	0.36 ± 0.01 [a]	0.21 ± 0.01	0.44 ± 0.17 [b]
	21.3–22.6			1.85–2.09	46.0–55.0	0.35–0.37	0.19–0.23	0.31–0.73

Life stage	Cu	Fe	Mn	Ni	Pb	Se	V	Zn
Hatchlings	47.2 ± 5.0 [b]	103 ± 71 [a]	2.67 ± 0.36 [a]	1.71 ± 0.46 [a]	< 0.46	1.81 ± 0.21 [b]	< 1.8	90.2 ± 8.1
	41.7–57.7	30.5–218	2.20–3.49	1.24–2.74	< 0.23–0.68	1.53–2.09	–	79.2–100
Juveniles	59.3 ± 3.1 [a]	49.1 ± 9.6 [ab]	2.29 ± 0.14 [ab]	1.36 ± 0.20 [ab]	0.33 ± 0.06	2.08 ± 0.11 [ab]	< 1.0	82.3 ± 6.8
	54.8–62.8	35.9–59.5	2.15–2.52	1.25–1.71	0.25–0.34	1.94–2.20	–	76.8–93.6
	67.2 ± 6.7 [a]	34.8 ± 4.0 [b]	2.14 ± 0.23 [bc]	1.23 ± 0.15 [bc]	0.43 ± 0.13	2.64 ± 0.05 [a]	< 0.6	88.2 ± 4.6
	57.7–75.5	28.6–38.7	1.87–2.46	1.01–1.42	0.28–0.58	2.58–2.69	–	85.0–96.0
	60.7 ± 7.6 [a]	25.3 ± 4.1 [c]	1.95 ± 0.10 [c]	1.06 ± 0.08 [c]	0.43 ± 0.11	2.04 ± 0.28 [ab]	< 0.8	87.3 ± 12.3
	50.0–71.1	21.0–31.2	1.87–2.09	0.95–1.13	0.34–0.60	1.76–2.40	–	73.9–104.9
	65.2 ± 5.1 [a]	25.0 ± 8.0 [bc]	1.85 ± 0.26 [bc]	1.05 ± 0.09 [c]	0.36 ± 0.08	2.26 ± 0.08 [a]	< 1.0	92.3 ± 2.2
	56.6–70.1	20.2–39.2	1.59–2.28	0.97–1.20	0.25–0.45	2.16–2.36	–	88.5–94.0

The different letters denote for each trace element, statistically significant differences (at *p*-value < 0.05) between sampling times ($a \neq b \neq c$).

Table 2: Soluble trace element proportion (mean ± SD; %) in whole juvenile *Sepia officinalis* during its two first months of life.

Life stage	Mantle length (mm)	Age	*N*	Ag	As	Cd	Co	Cr
Hatchlings	9.2 ± 0.5	0 days	9	n.d.	92.8 ± 1.8	n.d.	49.2 ± 5.9 ^{c}	62.1 ± 14.6 ^{b}
	8.4–9.8			n.d.	89.5–95.5	n.d.	43.1–60.8	43.6–83.7
Juveniles	10.3 ± 0.1	14 days	5	10.4 ± 1.7 ^{a}	93.9 ± 1.4	27.6 ± 5.5	68.1 ± 3.2 ^{b}	50.0 ± 17.2 ^{b}
	10.1–10.5			8.5–12.9	92.2–95.3	19.7–34.5	63.8–72.0	33.0–77.4
	11.7 ± 0.7	1 month	5	8.4 ± 1.3 ^{ab}	93.6 ± 1.4	25.4 ± 4.7	68.5 ± 3.5 ^{b}	68.0 ± 9.7 ^{ab}
	10.9–12.7			7.1–10.2	91.3–94.9	18.5–30.1	64.2–72.1	57.9–79.1
	16.4 ± 0.9	1.5 months	5	7.4 ± 0.6 ^{b}	92.4 ± 0.9	30.7 ± 3.3	74.9 ± 2.0 ^{a}	n.d.
	15.0–17.4			6.8–7.9	91.2–93.6	27.1–34.8	72.1–76.7	n.d.
	22.3 ± 1.3	2 months	5	7.8 ± 0.9 ^{ab}	92.7 ± 1.5	29.2 ± 1.6	70.3 ± 2.1 ^{b}	83.2 ± 9.9 ^{a}
	21.3–22.6			6.6–8.9	90.3–94.3	28.2–31.2	66.8–72.5	67.0–92.3

Life stage	Cu	Fe	Mn	Ni	Pb	Se	Zn
Hatchlings	27.3 ± 11.8 ^{b}	14.0 ± 12.8 ^{c}	35.6 ± 6.7	73.5 ± 9.7	n.d.	51.4 ± 3.6 ^{b}	14.8 ± 5.8 ^{b}
	11.0–39.8	2.4–37.4	22.5–44.4	56.9–82.6	n.d.	46.5–56.3	6.0–23.6
Juveniles	41.3 ± 7.5 ^{b}	31.9 ± 7.4 ^{b}	41.6 ± 3.7	73.5 ± 5.2	79.3 ± 2.8	62.0 ± 3.6 ^{a}	18.4 ± 2.9 ^{b}
	31.2–51.9	25.6–42.8	37.1–45.0	67.9–81.9	76.8–83.6	55.8–65.1	15.6–22.3
	40.8 ± 6.2 ^{b}	34.9 ± 4.8 ^{b}	41.3 ± 8.5	77.0 ± 3.3	83.4 ± 4.5	64.0 ± 2.0 ^{a}	20.2 ± 4.5 ^{b}
	33.8–49.7	31.3–43.1	28.0–50.1	74.3–80.9	79.0–87.5	62.1–66.9	14.9–25.0
	55.1 ± 5.1 ^{a}	48.6 ± 7.7 ^{a}	36.6 ± 7.9	78.6 ± 2.4	74.8 ± 6.7	63.5 ± 1.5 ^{a}	26.6 ± 4.0 ^{ab}
	47.3–61.0	37.8–55.9	22.8–41.4	75.7–81.5	69.1–85.8	60.9–65.1	23.4–32.3
	55.8 ± 0.7 ^{a}	35.7 ± 7.2 ^{ab}	28.2 ± 6.1	78.4 ± 0.9	75.1 ± 3.9	63.6 ± 2.0 ^{a}	30.0 ± 1.2 ^{a}
	55.1–56.8	24.6–44.0	21.0–37.1	77.3–79.2	69.4–80.2	60.5–66.0	28.6–31.7

The different letters denote for each trace element, statistically significant differences (at p-value < 0.05) between sampling times ($a \neq b \neq c$).

Table 3: Positive correlations between soluble concentrations of trace elements and metallothioneins measured in control juvenile cuttlefish between 2- and 8-week post-hatching. Upright and italic scripts correspond to Pearson and Spearman correlations, respectively. Underlined: $p < 0.001$; other: $p < 0.05$.

Elements								
Ag	+ Cd	+ Co	*+ Cu*	*+ Zn*				
As	*+ Cr*	*+ Fe*	*+ Mn*	*+ Ni*				
Cd	+ Ag	+ Co	+ Cu	+ Zn				
Co	+ Ag	+ Cd	+ Cu	+ Fe	*+ Ni*	+ Pb	+ Se	*+ Zn*
Cr	*+ As*							
Cu	*+ Ag*	+ Cd	+ Co	+ Zn	+ MTs			
Fe	*+ As*	+ Co	+ Mn	*+ Ni*				
Mn	*+ As*	+ Fe	*+ Ni*					
Ni	*+ As*	*+ Co*	*+ Fe*	*+ Mn*	+ Se			
Pb	+ Co							
Se	+ Co	+ Ni						
Zn	*+ Ag*	+ Cd	*+ Co*	+ Cu	*+ MTs*			
MTs	+ Cu	*+ Zn*						

Table 4: Positive correlations between insoluble concentrations of trace elements measured in control juvenile cuttlefish between 2- and 8-week post-hatching. Upright and italic scripts correspond to Pearson and Spearman correlations, respectively. Underlined: $p < 0.001$; other: $p < 0.05$.

Elements					
Ag	+ Cd	+ Co	+ Pb		
As	*+ Cr*	*+ Fe*	*+ Ni*	*+ Pb*	*+ Se*
Cd	+ Ag	*+ Co*	*+ Cu*	*+ Zn*	
Co	+ Ag	*+ Cd*	+ Cu	*+ Mn*	*+ Zn*
Cr	*+ As*	*+ Fe*	*+ Ni*	*+ Se*	
Cu	+ Cd	+ Co	*+ Zn*		
Fe	*+ As*	*+ Cr*	+ Mn		
Mn	*+ Co*	+ Fe	+ Ni		
Ni	*+ As*	*+ Cr*	+ Mn		
Pb	+ Ag	*+ As*			
Se	*+ As*	*+ Cr*			
Zn	*+ Cd*	*+ Co*	+ Cu		

Table 5: Zinc concentrations (mean ± SD in µg l^{-1} and µM) in seawater during the experiment.

Zn concentration	Control	Z1	Z2
µg l^{-1}	< 6.2	52 ± 3	184 ± 11
µM	< 0.1	0.80 ± 0.05	2.81 ± 0.16

Table 6: Trace metal concentrations (mean ± SD in $\mu g.g^{-1}$ dw) in prey (*C. crangon*) used during this study.

Fe	55.1 ± 25.0
Zn	43.2 ± 4.1
Cu	25.7 ± 5.6
As	15.7 ± 3.7
Mn	6.18 ± 3.49
Se	2.33 ± 0.73
Ni	1.56 ± 0.60
Ag	0.99 ± 0.13
Cd	0.25 ± 0.08
Cr	0.23 ± 0.18
Co	0.21 ± 0.04
Pb	0.17 ± 0.10
Hg	0.09 ± 0.02
V	< 0.39

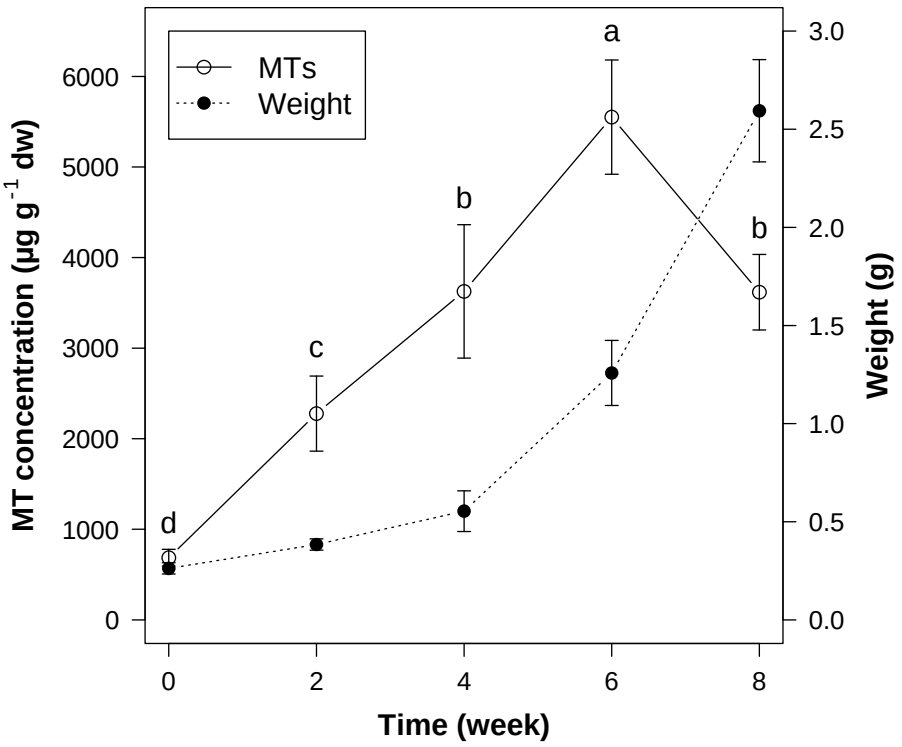

Figure 1: Metallothionein concentrations (mean ± SD; µg g^{-1} dw) in juvenile cuttlefish and associated weight (mean ± SD; g) during the 2 months post-hatching. Letters above the metallothionein concentrations indicate significant differences between groups (*p*-value < 0.05).

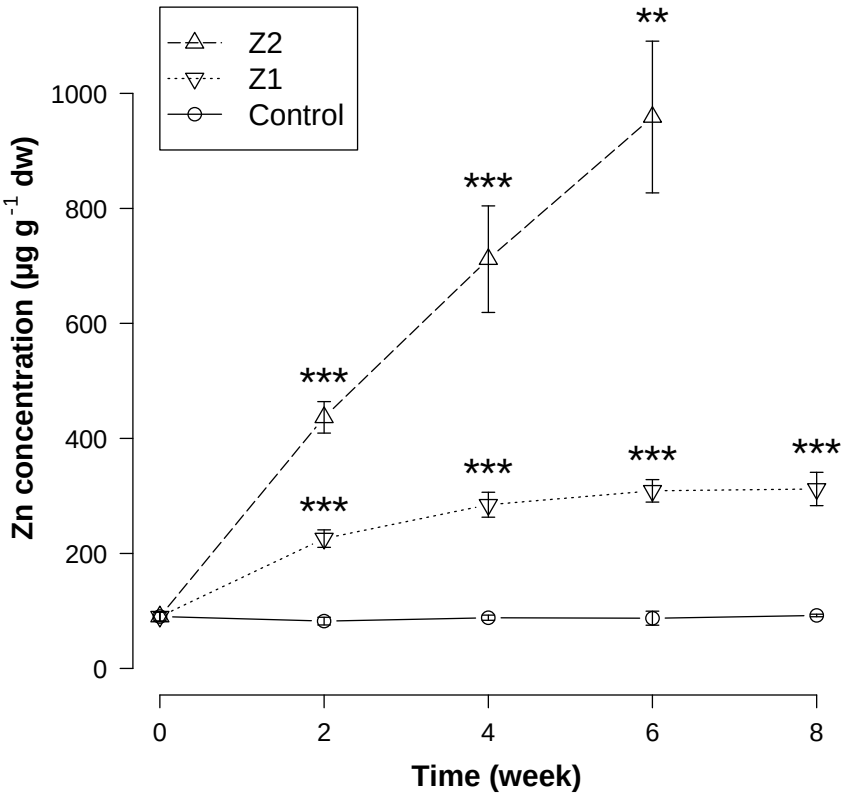

Figure 2: Total Zn concentrations (mean ± SD; µg g^{-1} dw) in juvenile cuttlefish reared in control and in 2 Zn concentrations (Z1, Z2). Asterisks correspond to significant differences compared to control (* < 0.05, ** < 0.01, *** < 0.001).

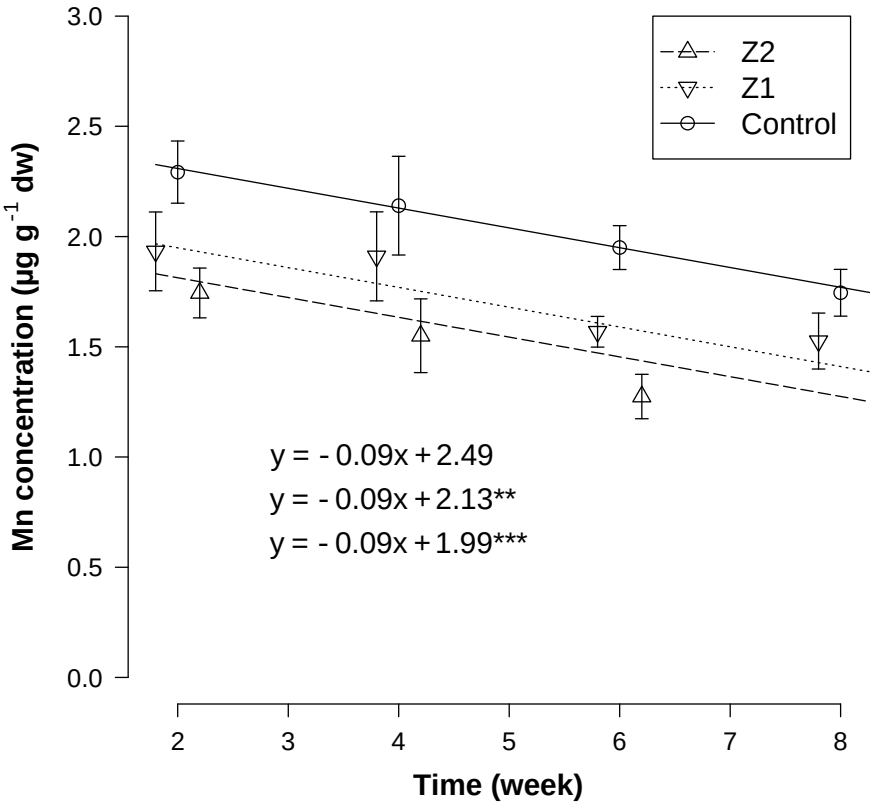

Figure 3: Total Mn concentrations (mean ± SD; µg g^{-1} dw) in juvenile cuttlefish reared in control and in two Zn concentrations (Z1, Z2). Asterisks correspond to significant differences compared to control (** < 0.01, *** < 0.001).

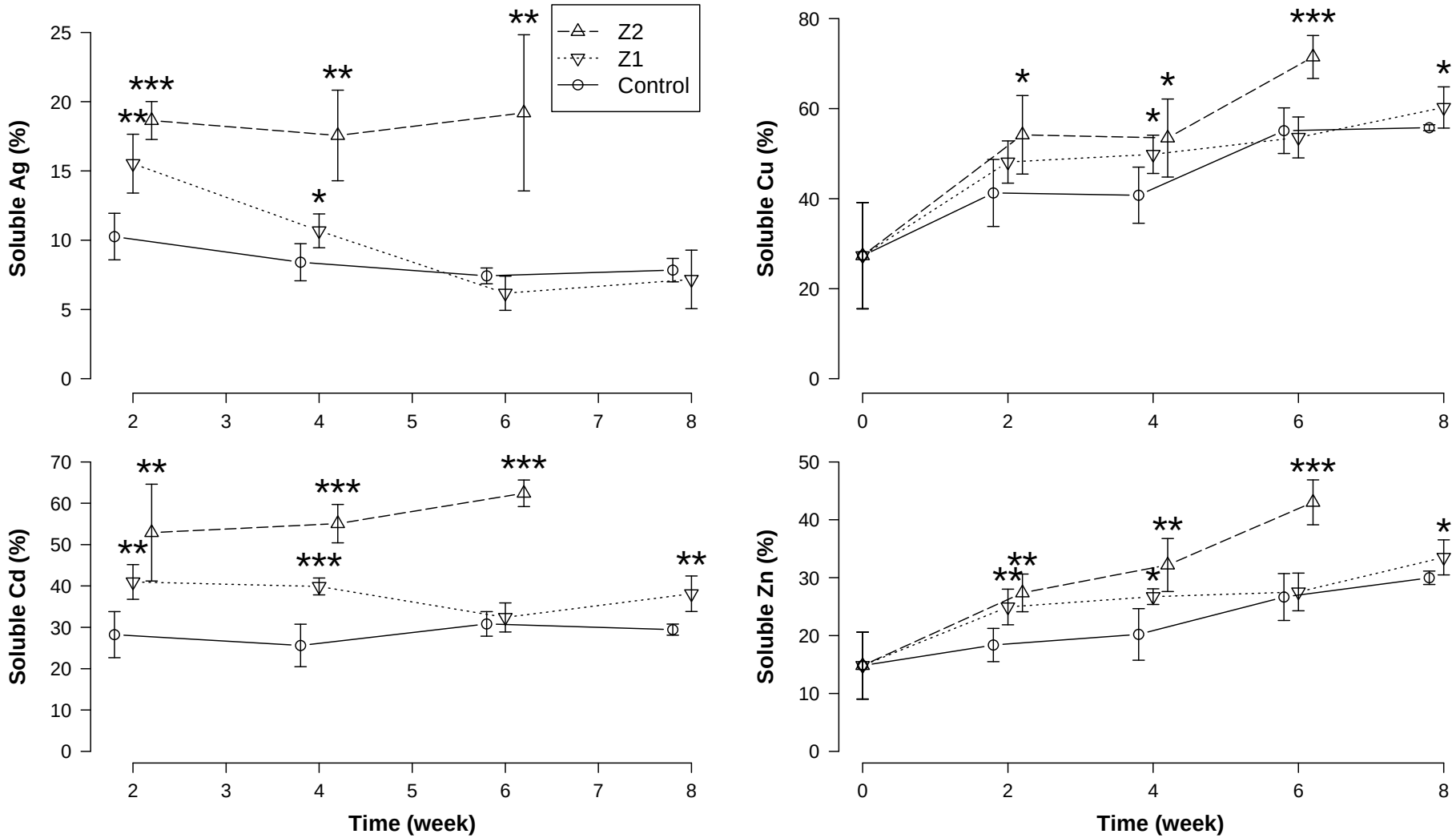

Figure 4: Soluble Ag, Cd, Cu and Zn proportions (mean ± SD; %) in juvenile cuttlefish reared in control and in 2 Zn concentrations (Z1, Z2). Asterisks correspond to significant differences compared to control (* < 0.05, ** < 0.01, *** < 0.001).

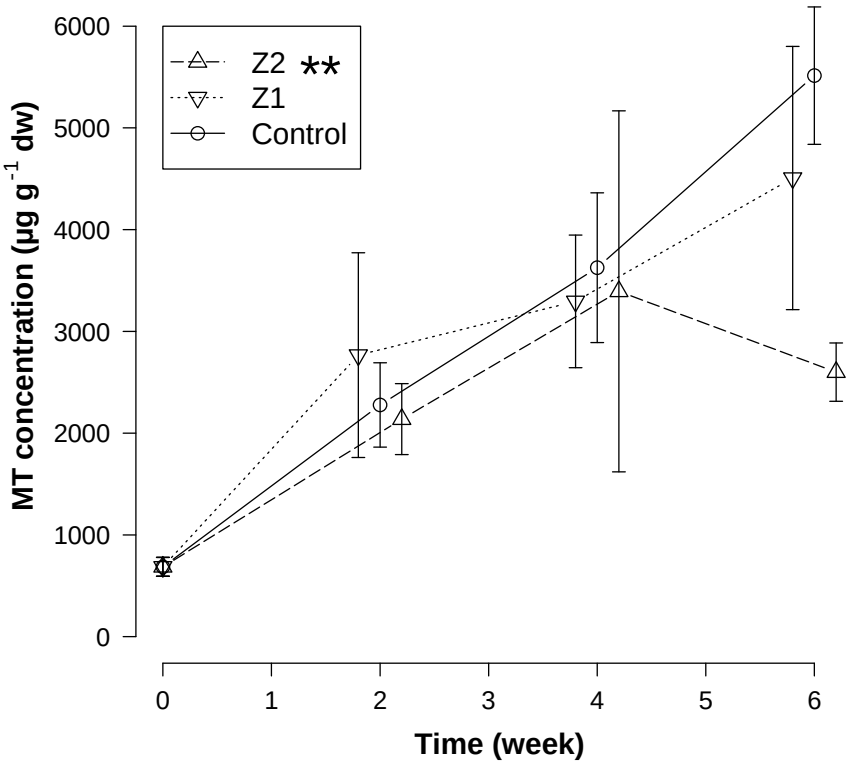

Figure 5: Metallothionein concentrations (mean ± SD; µg g^{-1} dw) in juvenile cuttlefish reared in control and in 2 Zn concentrations (Z1, Z2) during 6 week post-hatching. Asterisks correspond to significant differences of metallothionein changes over time compared to control (i.e. different slopes; ** < 0.01).

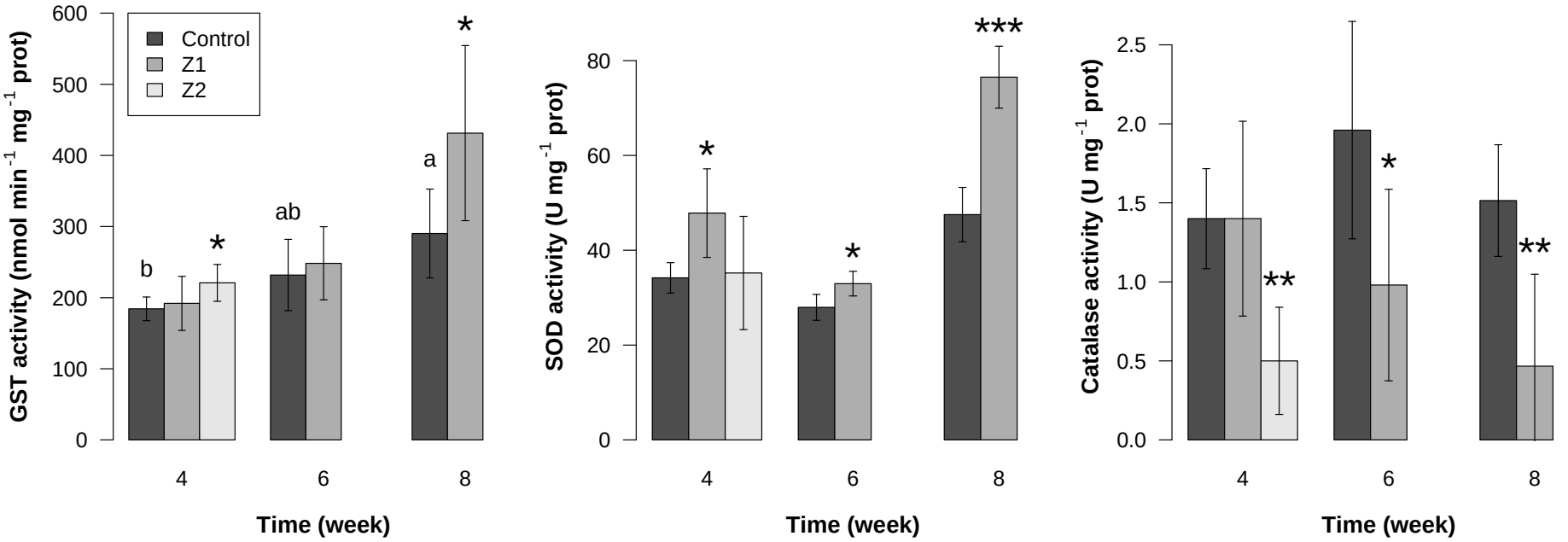

Figure 6: Glutathione S-transferase, superoxide dismutase and catalase activities (mean ± SD; respectively in nmol min^{-1} mg^{-1} prot, U mg^{-1} prot, and U mg^{-1} prot) in the digestive gland of juvenile cuttlefish in control, Z1 and Z2 conditions after 4-week post-hatching and in control and Z1 conditions after 6- and 8-week post-hatching. Letters above control indicate its change over time, and asterisks above Z1 or Z2 correspond to significant differences of activity compared to control (* < 0.05, ** < 0.01).

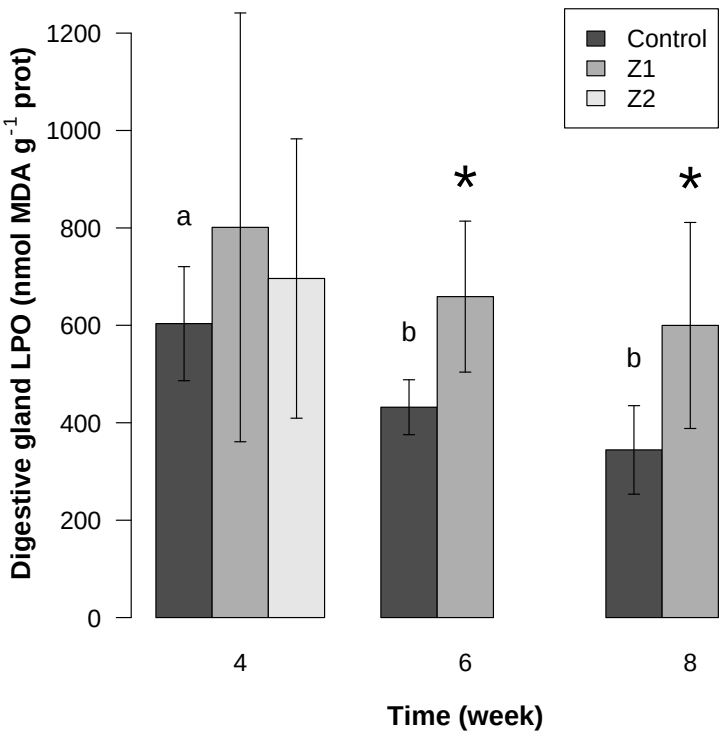

Figure 7: Lipid peroxidation (mean ± SD; nmol MDA g^{-1} prot) in the digestive gland of juvenile cuttlefish in control, Z1 and Z2 conditions after 4-week post-hatching and in control and Z1 conditions after 6- and 8-week post-hatching. Letters above control indicate its change over time, and asterisks above Z1 correspond to significant differences of activity compared to control (*<0.05).